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Light Microscopy and Phylogenetic Analyses of *Chlamydomonas* Species (Volvocales, Chlorophyceae). I. *Chlamydomonas pseudomacrostigma* and *Chlamydomonas media*

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Two species of *Chlamydomonas*, *Chlamydomonas pseudomacrostigma* L. Ş. Péterfi ex H. Ettl and *Chlamydomonas media* G. A. Klebs, were examined using light microscopy and the molecular phylogeny of 18S *r*RNA gene sequences. The molecular phylogeny clearly showed that both species belong to the clade *Monadinia* and are closely related to *Chlamydomonas monadina* F. Stein and *Chloromonas subdivisa* (Pascher & Jahoda) Gerloff & H. Ettl. Based on detailed light microscopy, the chloroplasts of *C. pseudomacrostigma* were shown to be deep-cup-shaped with a single parietal pyrenoid (*Chlamydella*-type). Both light microscopy and 18S *r*RNA gene phylogeny also revealed that an additional strain previously identified as *Chlamydomonas monadina* actually belong to *C. pseudomacrostigma*. A newly isolated Japanese strain of *C. media* was phylogenetically separated from "*C. media*" SAG 10.87, which was further distinguished from *C. media* by the morphology of the stigma.

Key words: 18S *r*RNA, *Chlamydomonas media*, *Chlamydomonas pseudomacrostigma*, light microscopy, molecular phylogeny, phycology

Chlamydomonas Ehrenb. (Volvocales, Chlorophyceae), with 400 to 600 species, is the largest polyphyletic genus of unicellular freshwater green algae (Ettl 1976, 1983, Pröschold *et al.* 2001, Nakada *et al.* 2008). Because many volvocalean genera, families and even suborders have been shown to be polyphyletic (e.g. Buchheim *et al.* 1990, 1996, 1997, Nozaki *et al.* 2000, Pröschold *et al.* 2001, Pröschold & Leliaert 2007), there is currently no useful Linnean taxonomic system within the order Volvocales. Instead, Nakada *et al.* (2008) proposed a phylogenetic classification following PhyloCode (Cantino & de Queiroz 2010), thus making it possible to determine the affiliation of a volvocalean species based solely on its phylogenetic position.

As a first step towards reclassification of the highly confusing *Chlamydomonas* species, it is necessary to determine the phylogenetic affiliations of each species based on culture strains that are morphologically well characterized. In this study, strains of *Chlamydomonas pseudomacrostigma* L. Ş. Péterfi ex H. Ettl and *Chlamydomonas media* G. A. Klebs from Japan were newly isolated and characterized using light microscopy and 18S *r*RNA gene phylogeny. They were then compared with closely related strains and strains previously identified as *C. pseudomacrostigma* SAG 31.86, "*C. media*" SAG 10.87 and "*Chlamydomonas monadina* F. Stein" SAG 8.87.

Materials and Methods

Isolations and cultivations

Two strains, HcCl-5-3 and TkS0811B3, were newly isolated from rewetted soil samples collected from Ibaraki, Japan (July 6, 2004), and Tsuruoka, Yamagata, Japan (May 29, 2008), respectively, according to the pipette-washing method (Pringsheim 1946). Both strains were deposited in the Microbial Culture Collection at the National Institute for Environmental Studies (NIES; http://mcc.nies.go.jp/top.jsp) as NIES-2743 (= HcCl-5-3) and NIES-2744 (= TkS0811B3). Other strains (SAG 8.87, 10.87 and 31.86) were obtained from Sammlung von Algenkulturen at the University of Göttingen (SAG; http://www.epsag.uni-goettingen.de/). Culture maintenance of the strains was as described by Nakada & Nozaki (2007) in AF-6 medium (Kato 1982, modified as in Andersen *et al.* 2005) at 20°C.

Observations

Light microscopy was carried out using a Leica DM2500 microscope equipped with Nomarski interference optics (Leica, Wetzlar, Germany) and an Olympus DP71 digital camera (Olympus, Tokyo, Japan). Observations were performed using 7-day-old cultures unless otherwise stated.

DNA sequencing

The 18S *r*RNA gene sequences of four strains (HcCl-5-3, TkS0811B3, SAG 10.87 and SAG 31.86) were determined (AB694003–AB694006). DNA extraction, polymerase chain reaction (PCR) and sequencing were performed as described previously (Nakada *et al.* 2010a). The primers for PCR and sequencing were 18S-FA, RB, FC, RD, FE, RF, FA2 and RB2 (Nakazawa & Nozaki 2004, Nakada *et al.* 2007, 2010b).

Phylogenetic analyses

Based on preliminary analyses, 18S *r*RNA gene sequences obtained from the strains HcCl-5-3, TkS0811B3, SAG 10.87 and SAG 31.86 were affiliated to the clade *Monadinia* (Nakada *et al.* 2008), and unambiguously aligned with the available sequences of *Monadinia*. Sequences of the clade *Phacotinia* and *Moewusinia* were used as an outgroup (Nakada *et al.* 2008).

Bayesian phylogenetic analyses using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003, 1,000,000 generations of Markov chain Monte Carlo iterations, with the first 25% discarded as burn-in), and bootstrap analyses (Felsenstein 1985) of maximum likelihood (100 replications, nearest neighbor interchange heuristic searches started with a neighbor joining tree), maximum parsimony (1,000 replications) and neighbor-joining (Saitou & Nei 1987; 1,000 replications using Jukes and Cantor distances) analyses using PAUP* 4.0b10 (Swofford 2002) were performed as described by Nakada & Nozaki (2009). The evolutionary models for Bayesian inference (GTR + I + G) and maximum likelihood analysis (TrN + I + G) were selected using MrModeltest 2.2 (Nylander 2004) and Modeltest 3.7 (Posada & Crandall 1998), respectively.

Results and Discussion

Based on the molecular phylogeny and light microscopy results (see *Remarks* for each species), the strains TkS0811B3, "*Chlamydomonas monadina*" SAG 8.87 and *C. pseudomacrostigma* SAG 31.86 were (re)identified as *C. pseudomacrostigma*. The strain HcCl-5-3 was identified as *C. media* while "*C. media*" SAG 10.87 was determined not to be *C. media* and treated instead as *Chlamydomonas* sp. Additionally, epitypes for the types of *C. pseudomacrostigma* and *C. media* were designated with the figures based on the newly characterized strains.

Chlamydomonas pseudomacrostigma L. Ş. Péterfi ex H. Ettl (TkS0811B3, SAG 8.87 and SAG 31.86; Figs. 1 & 2)

Holotype. Bourrelly (1951), figs. 33–34 (France. A pond of Villebon, April.1948).

Epitype. Fig. 1 (**designated here**), based on the culture strain TkS0811B3 (= NIES-2744; isolated from Tsuruoka, Yamagata, Japan, 29. May.2008).

Synonymy. *Chlamydomonas pseudomacrostigma* L. Ş. Péterfi ex H. Ettl in Nova Hedwigia Beih. 49: 358. 1976 ≡ *Chlamydomonas macroplastida* J. W. G. Lund f. *macrostigma* Bourr. in Hydrobiologia. 3: 256. 1951. — Type: Bourrelly (1951), figs. 33–34 (holotype). Fig. 1 (epitype, **here designated**). [–*Chlamydomonas pseudomacrostigma* L. Ş. Péterfi in Nova Hedwigia 16: 223. 1968. nom. nud. (lacking direct reference to the original description by Bourrelly 1951)]

= *Chlamydomonas monadina* F. Stein var. *charkoviensis* Korshikov in Vyznachnyk prisnovodnykh vodorosteĭ URSR 4: 76. 1938. — Type: Korshikov (1938), fig. 30a (holotype; erroneously cited as “fig. 30b” in Korshikov 1938). [–*Chlamydomonas cingulata* Pascher var. *charkowiensis* Korshikov in Pascher, Süsswasserflora 4: 272. 1927. nom. nud. (lacking description and diagnosis, and name not accepted). –*Chlamydomonas proboscigera* Korshikov var. *charkowiensis* (Korshikov) L. Ş. Péterfi in Nova Hedwigia 16: 222. 1968. nom. nud. (lacking reference to the original description by Korshikov 1938)]

Description (*based on TkS0811B3, SAG 8.87*

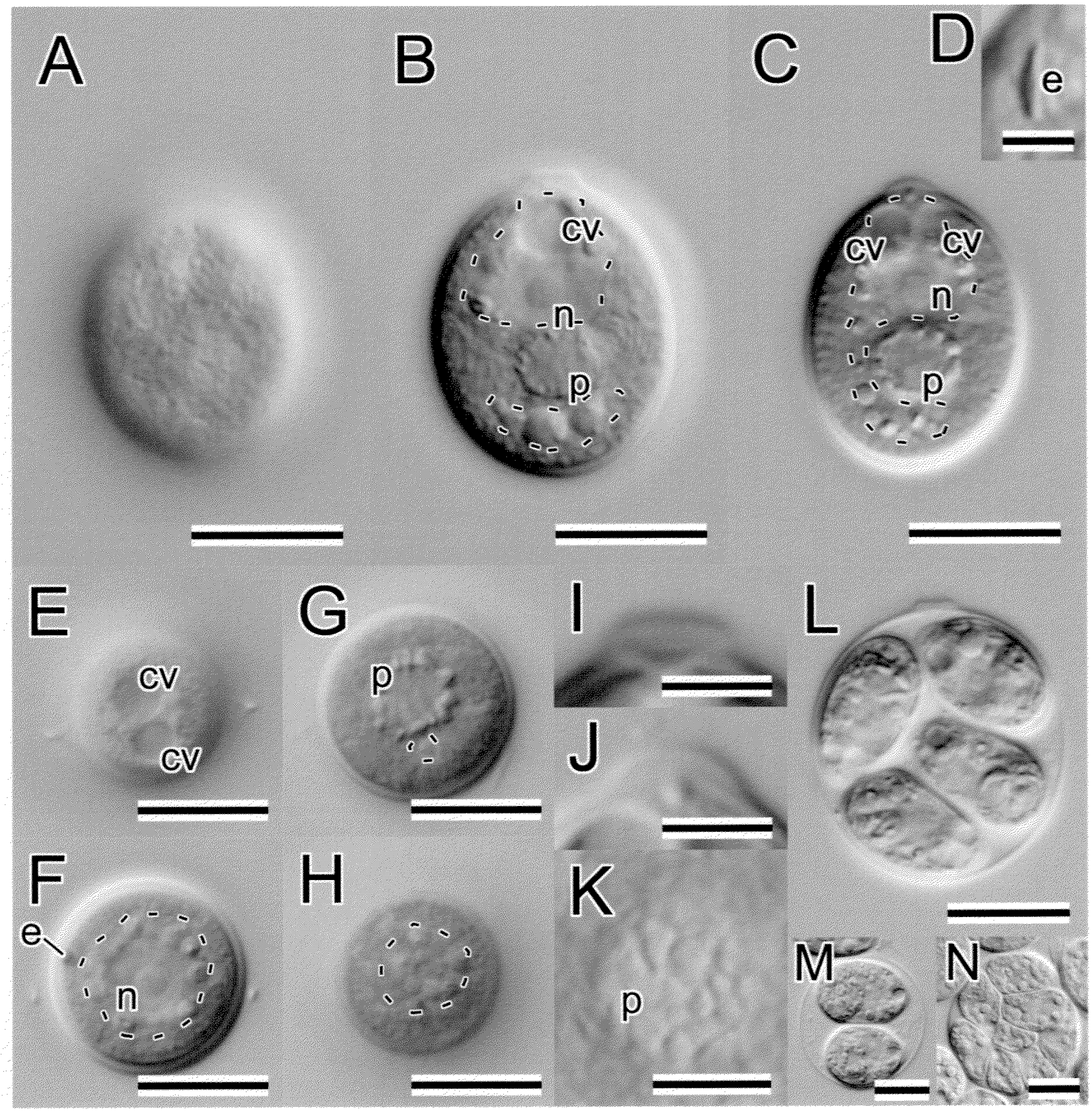

FIG. 1. *Chlamydomonas pseudomacrostigma* L.Ş. Péterfi ex H. Ettl (TkS0811B3 = NIES-2744). Chloroplast cavities are indicated by broken lines. A: Surface view of a vegetative cell. B: Optical section of the same cell in front view. C: Optical section in side view. D: Stigma. E–H: Successive optical sections of a cell in top view (from top to bottom). I: Papilla in front view. J: Papilla in side view. K: Surface of a pyrenoid. L–N: Zoosporangia (L, N: 4-day-old culture, M: 3-day-old culture). cv, contractile vacuole; e, stigma; n, nucleus; p, pyrenoid. Scale bars = 10 μm (A–C, E–H, L–N), 5 μm (K), 3 μm (D, I, J).

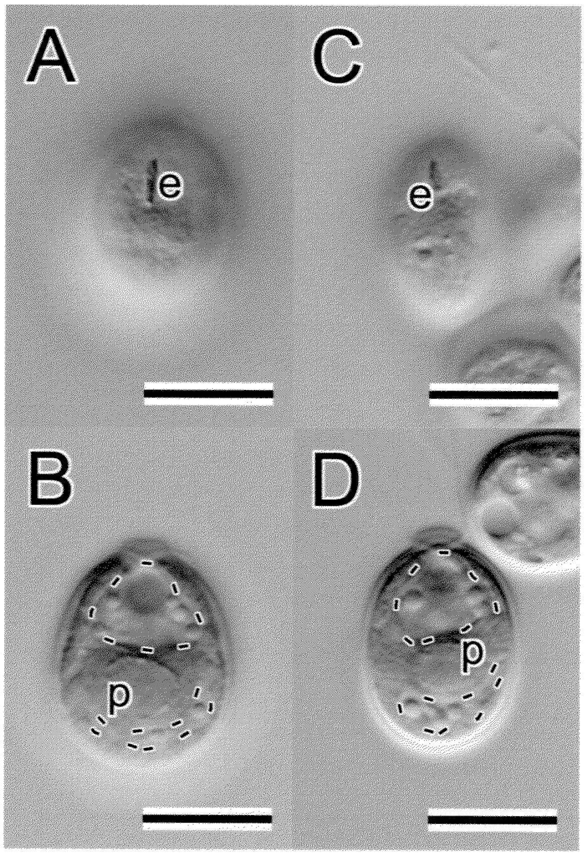

FIG. 2. *Chlamydomonas pseudomacrostigma* L.Ş. Péterfi ex H. Ettl (A, B: SAG 8.87, C, D: SAG 31.86). Chloroplast cavities are indicated by broken lines. A, C: Surface views of vegetative cells. B, D: Optical section of vegetative cells. For abbreviations, see the legend to Fig 1. Scale bars = 10 μm.

and SAG 31.86). Vegetative cells ellipsoid to ovoid, sometimes slightly asymmetrical with a less convex ventral surface (Figs. 1A–C & 2). Cell wall thick, with angular keel-like papilla on the front (Figs. 1B, I, J & 2B, D). Flagella two, as long as the cell. Chloroplast essentially deep-cup-shaped with a single parietal pyrenoid in the middle region of the cell (*Chlamydella*-type; Figs. 1B, C, E–G & 2B, D). Chloroplast highly developed, thickened, in pyrenoid region, with a cavity consisting of a small circular space between the pyrenoid and periphery of the chloroplast in cross section (Fig. 1G). Chloroplast surface relatively smooth (Figs. 1A & 2A, C). Pyrenoid large ellipsoid, half the width of the cell, central, covered with numerous starch granules, sometimes in a helical arrangement (Fig. 1B, C, G, K). Stigma linear, located in the anterior portion of the chloroplast (Figs. 1D & 2A, C). Nucleus in front cavity of chloroplast (Fig. 1B, C, F). Contractile vacuoles two, in apical region (Fig. 1B, C, E). Cells 14–25 μm long, 9–18 μm wide. Asexual reproduction by usually four, rarely two or eight zoospores (Fig. 1L–N).

Phylogeny. In the Bayesian tree of the clade *Monadinia* (Fig. 5), *Chlamydomonas pseudomacrostigma* TkS0811B3, SAG 8.87, SAG 31.86 and CCAP 11/46 formed a clade (1.00 posterior probability [PP] and 99–100% bootstrap proportions [BPs]). The 18S *r*RNA gene sequences of the latter three strains were identical, and differed from strain TkS0811B3 by three bases among the examined 1691 bases. The strains of *C. pseudomacrostigma* were sister to *C. monadina* SAG 31.72 (1.00 PP and 85–93% BPs).

Distribution. France (Bourrelly 1951), Romania (Péterfi 1968), Ukraine (Korshikov 1938, Massjuk *et al*. 2011), United Kingdom (SAG 8.87, SAG 31.86 and CCAP 11/46; SAG, http://www.epsag.uni-goettingen.de/; CCAP, http://www.ccap.ac.uk/) and Japan (TkS0811B3).

Strains. TkS0811B3 (=NIES-2744), SAG 8.87 (previously identified as "*Chlamydomonas monadina*"), SAG 31.86 and CCAP 11/46 ("*Chlamydomonas* sp.").

Remarks. The morphology of the culture strains was essentially the same as *Chlamydomonas pseudomacrostigma* described by Bourrelly (1951) and Péterfi (1968) in that they have ellipsoid cells with an angular papilla, an anterior nucleus, and a chloroplast with an anterior linear stigma (Figs. 1 & 2), but the structure of the chloroplast was different. According to Bourrelly (1951) and Péterfi (1968), *C. pseudomacrostigma* has a cup-shaped chloroplast with a basal pyrenoid (*Euchlamydomonas*-type), while the Japanese and British strains had a deep-cup-shaped chloroplast with a parietal pyrenoid (*Chlamydella*-type). However, the chloroplast cavity posterior to the pyrenoid was narrow and inconspicuous in the examined strains (Figs. 1B, C & 2B, D) and thus may have been overlooked by Bourrelly (1951) and Péterfi (1968).

The morphology of the strains of *Chlamydomonas pseudomacrostigma*, TkS0811B3, SAG

8.87 and SAG 31.86 (Figs. 1 & 2), was almost indistinguishable from the illustration of "*C. monadina* var. *charkoviensis*" by Korshikov (1938). However, the flagella of the examined strains were as long as the cell, unlike in the holotype in which they are shorter than the body, but the relative flagellar length is not stable through cell growth, and does not represent a species level difference.

Cells of *Chlamydomonas monadina* var. *monadina* have a half-ring shaped pyrenoid, but the strains TkS0811B3, SAG 8.87 and SAG 31.86 had an ellipsoid pyrenoid (Figs. 1B, C, G & 2B, D) and are phylogenetically separated from *C. monadina* SAG 31.72 (Fig. 5). Therefore, *C. monadina* var. *charkoviensis* is here separated from *C. monadina* and recognized at the species level as *C. pseudomacrostigma*. Although the morphology of strain CCAP 11/46, labeled *Chlamydomonas* sp., was not examined in this study, its 18S rRNA gene sequence was identical to that of SAG 8.87 and SAG 31.86, and it is thus included within *C. pseudomacrostigma*.

Among the species of *Chlamydomonas*, *Chlamydomonas pachychlamys* Pascher & Jahoda, *Chlamydomonas eupapillata* F. Moewus, *Chlamydomonas trulla* Pascher and *Chlamydomonas pseudoelliptica* Bourr. have ellipsoid to ovoid cells with keel-like or abrupt papillae and a smooth chloroplast with a linear stigma and a single pyrenoid (Ettl 1983). However, *C. pachychlamys* has smaller cells (8–14 μm), *C. eupapillata* has a posterior stigma, *C. trulla* has a dorsal not transversely central pyrenoid, and the nucleus of *C. pseudoelliptica* is longitudinally medial or more posterior. Additionally, *C. monadina* var. *seligeriensis* Korshikov is similar to *C. pseudomacrostigma*. Korshikov (1938) distinguished *C. pseudomacrostigma* (as *C. monadina* var. *charkoviensis*) from *C. monadina* var. *seligeriensis* based primarily on the position of the stigma (*C. monadina* var. *seligeriensis* has a posterior stigma). Although Ettl (1976) considered *C. monadina* var. *seligeriensis* to be an ecotype of "*C. proboscigera* var. *charkowiensis*," the former variety is not included in the synonymy here, because cells with a posterior stigma were not observed among the strains of *C. pseudomacrostigma*.

Boldina (1996) examined the ultrastructure of the pyrenoid of *Chlamydomonas pseudomacrostigma* CCAP 11/82 (= SAG 31.86), and found it to be associated with fragmented starch granules and a matrix dissected by two-layered flattened thylakoids (Boldina 1996). Konstantinova & Boldina (2000) and Boldina (2011) called this kind of pyrenoid a Type III pyrenoid. Rosowski & Hoshaw (1988) examined *C. monadina* UTEX 760 and confirmed that the pyrenoid was also Type III, supporting the close relationship between *C. pseudomacrostigma* and *C. monadina*.

Chlamydomonas media G. A. Klebs var. **media** (HcCl-5-3; Fig. 3)

Holotype. Klebs (1896), fig. 12 (culture strain isolated from Basel, Switzerland, in autumn, 1895).

Epitype. Fig. 3 (**designated here**), based on the culture strain HcCl-5-3 (= NIES-2743; isolated from Ibaraki, Japan, 6.July.2004).

Synonymy. *Chlamydomonas media* G. A. Klebs var. *media* in Fortpfl. Alg. Pilz.: 424. 1896. — Type: Klebs (1896), fig. 12 (holotype). Fig. 3 (epitype, **here designated**).

= *Chlamydomonas media* var. *eustigma* Gerloff in Arch. Protistenkunde 94: 362. 1940. — Type: Gerloff (1940), figs. 30–31 (holotype).

Description (*based on HcCl-5-3*). Vegetative cells asymmetrical obovoid, ellipsoid, short cylindrical to ovoid with almost straight ventral surface (Fig. 3A–C). Cell wall thin, with a slightly rounded keel-like papilla in front (Fig. 3B, J, K). Flagella two, as long as cell. Chloroplast deep-cup-shaped, with a single parietal pyrenoid in middle region (*Chlamydella*-type; Fig. 3B–I). Chloroplast semi-circumferentially thickened in pyrenoid region, broadly crescent-shaped in cross-section (Fig. 3G, H). Chloroplast surface relatively smooth (Fig. 3A). Pyrenoid large, ellipsoid, half as wide as cell, on dorsal surface, covered with numerous starch granules (Fig. 3B, C, G, H, L). Stigma linear, located in anterior portion of chloroplast (Fig. 3A). Nucleus located near

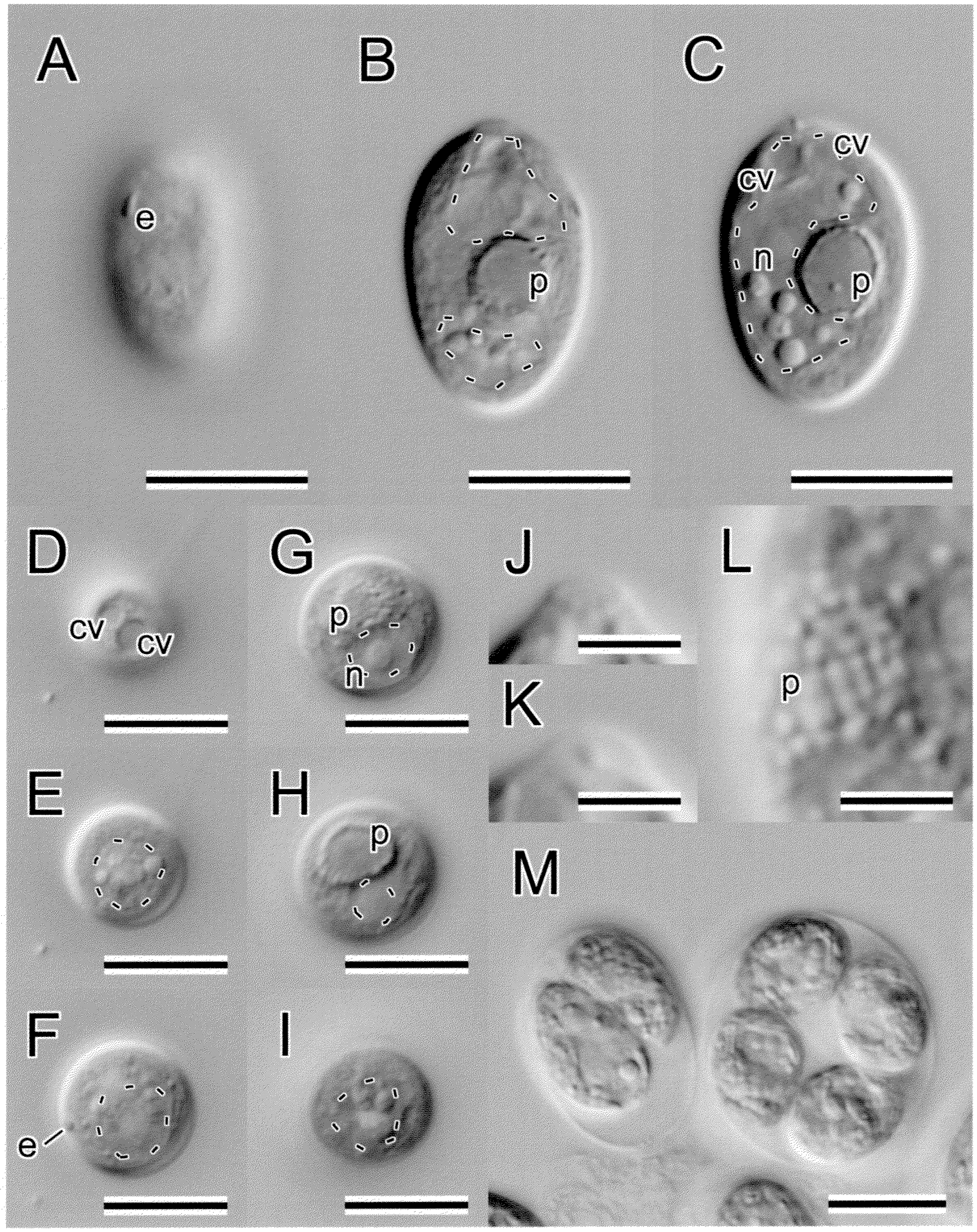

FIG. 3. *Chlamydomonas media* G.A. Klebs var. *media* (HcCl-5-3 = NIES-2743). Chloroplast cavities are indicated by broken lines. A: Surface view of a vegetative cell. B: Optical section in front view. C: Optical section in side view. D–I: Successive optical sections of a cell in top view (from top to bottom). J: Papilla in front view. K: Papilla in side view. L: Surface of a pyrenoid. M: Zoosporangia. For abbreviations, see the legend to Fig 1. Scale bars = 10 μm (A–I, M), 3 μm (J–L).

and often slightly above pyrenoid (Fig. 3C, G). Contractile vacuoles two, in apical region (Fig. 3C, D). Cells 13–20 μm long, 7–13 μm wide. Asexual reproduction by usually two, rarely four, zoospores (Fig. 3M).

Phylogeny. In the Bayesian tree of the clade *Monadinia* (Fig. 5), *Chlamydomonas media* HcCl-5-3 formed a clade with *C. pseudomacrostigma*, *C. monadina* SAG 31.72, *Chloromonas subdivisa* (Pascher & Jahoda) Gerloff & H. Ettl SAG 67.72 and *Chlamydomonas* sp. ("*Chlamydomonas media*") SAG 10.87 (1.00 PP and 75–91% BPs). The sister group of *C. media* was not resolved in these analyses; however, the sister relationship between *C. media* HcCl-5-3 and *Chlamydomonas* sp. SAG 10.87 (previously labeled "*C. media*") was rejected (< 0.05 PP and < 5% BPs).

Distribution. Common in Europe, including the former USSR (Ettl 1976, 1983, Massjuk *et al.* 2011), and Japan (HcCl-5-3).

Strain. HcCl-5-3 (=NIES-2743)

Remarks. Pascher (1927) established *Chlamydomonas media* var. *minor* Pascher based on its smaller size (< 12 μm long) in comparison with the type variety (14–20 μm long, according to Klebs 1896). Although Gerloff (1940) established another variety, *C. media* var. *eustigma*, its diagnostic characteristics (a flatter papilla, larger stigma, irregular chloroplast and pyrenoid with thicker starch plates) are trivial. The original drawings (Gerloff 1940) are almost indistinguishable from the type variety (Klebs 1896, fig. 12; Gerloff 1940, fig. 46). *Chlamydomonas media* var. *eustigma* is therefore treated here as a synonym of *Chlamydomonas media* var. *media*.

Among the species of *Chlamydomonas* having a *Chlamydella*-type chloroplast, three taxa (*Chlamydomonas pseudogloeogama* Gerloff, *Chlamydomonas moewusii* Gerloff var. *moewusii* and *Chlamydomonas moewusii* var. *microstigmata* (J. W. G. Lund) H. Ettl) are similar to *C. media* in having somewhat ellipsoid cells with rounded ends and a similar size range (13–20 μm), a homogenous chloroplast with a smooth surface and a long elliptic or linear stigma in the anterior region, a nucleus near or anterior to the pyrenoid, and rounded conical or keel-like papillae. *Chlamydomonas pseudogloeogama*, however, has slightly smaller cells (11–15 μm; Ettl 1983) and is

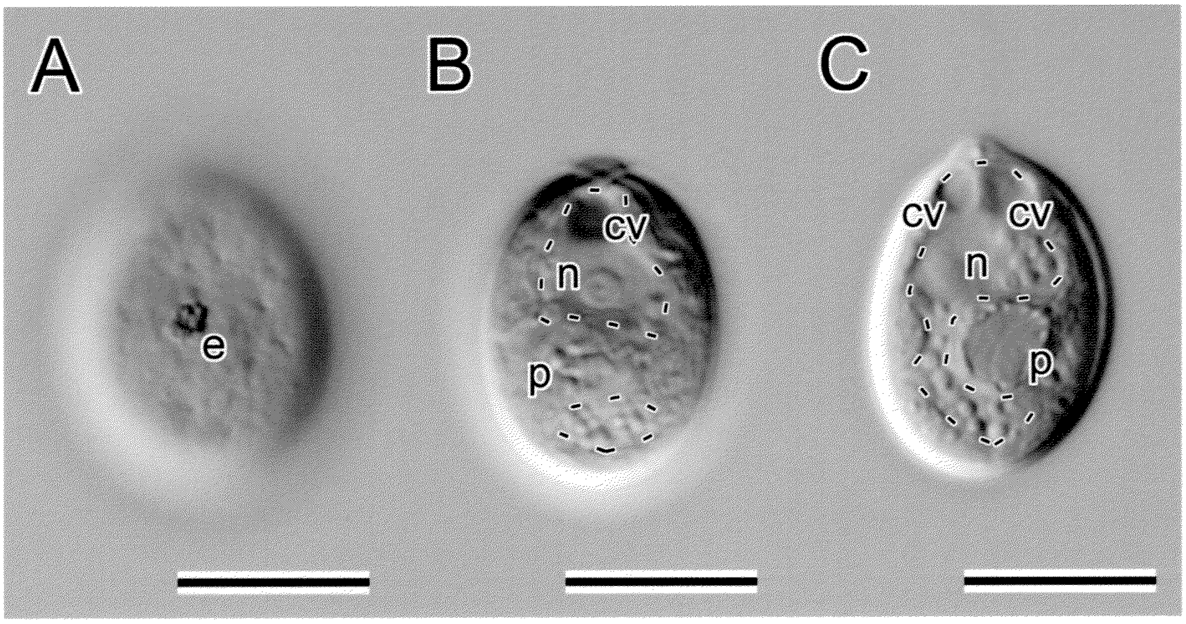

FIG. 4. *Chlamydomonas* sp. (SAG 10.87). Chloroplast cavities are indicated by broken lines. A: Surface view of a vegetative cell. B, C: Optical section of vegetative cells (B, front view; C, side view). For abbreviations, see the legend to Fig 1. Scale bars = 10 μm.

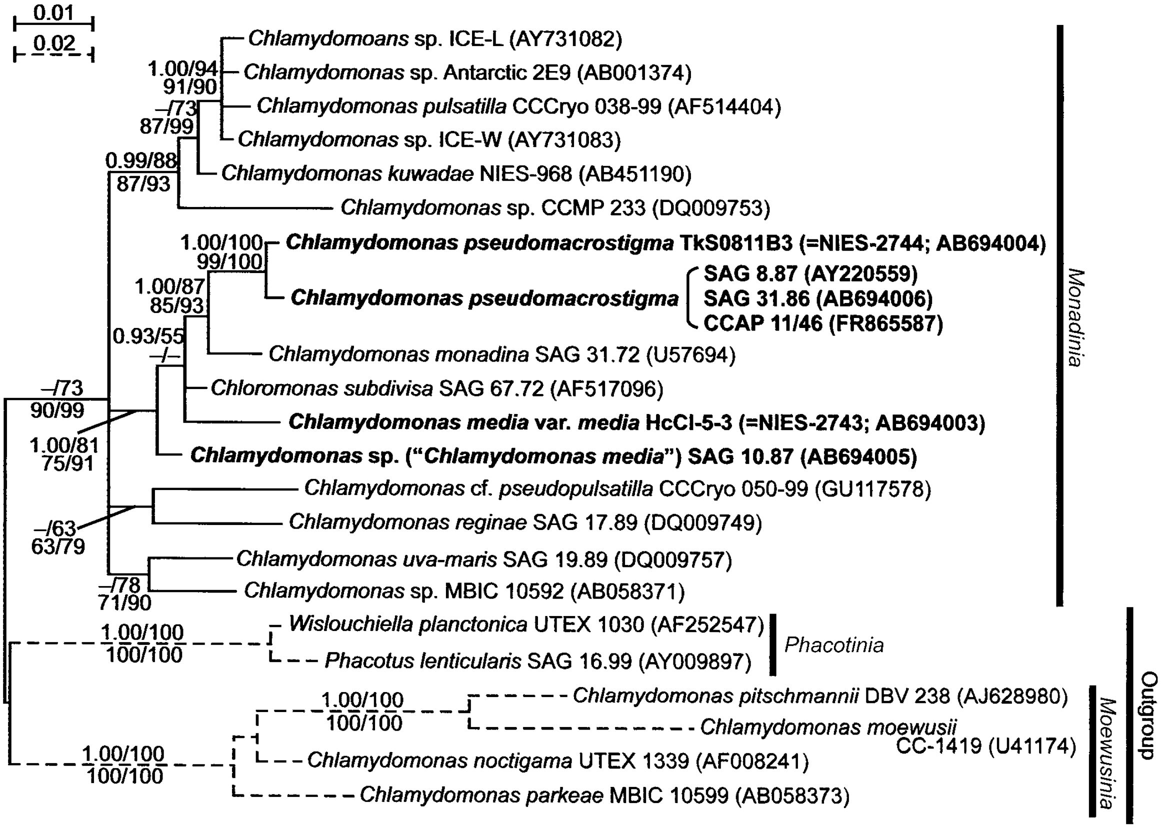
0.01
0.02
Chlamydomoans sp. ICE-L (AY731082)
Chlamydomonas sp. Antarctic 2E9 (AB001374)
Chlamydomonas pulsatilla CCCryo 038-99 (AF514404)
Chlamydomonas sp. ICE-W (AY731083)
Chlamydomonas kuwadae NIES-968 (AB451190)
Chlamydomonas sp. CCMP 233 (DQ009753)
Chlamydomonas pseudomacrostigma TkS0811B3 (=NIES-2744; AB694004)
Chlamydomonas pseudomacrostigma
SAG 8.87 (AY220559)
SAG 31.86 (AB694006)
CCAP 11/46 (FR865587)
Chlamydomonas monadina SAG 31.72 (U57694)
Chloromonas subdivisa SAG 67.72 (AF517096)
Chlamydomonas media var. media HcCl-5-3 (=NIES-2743; AB694003)
Chlamydomonas sp. ("Chlamydomonas media") SAG 10.87 (AB694005)
Chlamydomonas cf. pseudopulsatilla CCCryo 050-99 (GU117578)
Chlamydomonas reginae SAG 17.89 (DQ009749)
Chlamydomonas uva-maris SAG 19.89 (DQ009757)
Chlamydomonas sp. MBIC 10592 (AB058371)
Wislouchiella planctonica UTEX 1030 (AF252547)
Phacotus lenticularis SAG 16.99 (AY009897)
Phacotinia
Chlamydomonas pitschmannii DBV 238 (AJ628980)
Chlamydomonas moewusii CC-1419 (U41174)
Chlamydomonas noctigama UTEX 1339 (AF008241)
Chlamydomonas parkeae MBIC 10599 (AB058373)
Moewusinia
Outgroup
Monadinia
1.00/94
91/90
–/73
87/99
0.99/88
87/93
1.00/100
99/100
1.00/87
85/93
0.93/55
–/–
–/73
90/99
1.00/81
75/91
–/63
63/79
–/78
71/90
1.00/100
100/100
1.00/100
100/100
1.00/100
100/100

FIG. 5. Bayesian phylogenetic tree of the clade *Monadinia* (Volvocales) based on 18S *r*RNA gene sequences showing the phylogenetic positions of *Chlamydomonas pseudomacrostigma*, *Chlamydomonas media* var. *media* and *Chlamydomonas* sp. SAG 10.87 (shown in bold). Sequences of the clades *Phacotinia* and *Moewusinia* were used as the outgroup. The corresponding posterior probabilities (≥ 0.90; top left) and the bootstrap proportions (≥ 50%) from maximum likelihood (top right), maximum parsimony (bottom left) and neighbor-joining (bottom right) analyses are shown by the branches. Branch lengths and the scale bar represent the expected number of nucleotide substitutions per site. Accession numbers of the International Nucleotide Sequence Databases for each gene are indicated in parenthesis.

phylogenetically affiliated to *Moewusinia* (Nakada *et al.* 2008). The cells of *C. moewusii* var. *moewusii* are slightly smaller (9–16 μm) than those of *C. media* (13–20 μm), and the stigma of *C. moewusii* var. *microstigmata* is apparently smaller than *C. media.* Phylogenetically, *C. moewusii* is affiliated to the clade *Moewusinia* (Buchheim *et al.* 1990, Nakada *et al.* 2008).

Although the strain SAG 10.87 was labeled "*Chlamydomonas media*" (http://www.epsag.uni-goettingen.de/), it is phylogenetically distant from *C. media* HcCl-5-3 (Fig. 5). Strain SAG 10.87 was similar to HcCl-5-3 in that it has ellipsoid vegetative cells with a keel-like papilla and a *Chlamydella*-type pyrenoid (Figs. 3 & 4). Both strains, however, were clearly distinguished from each other by their stigmata. While strain HcCl-5-3 had a linear stigma in the anterior portion of the cell (Fig. 3A), strain SAG 10.87 had a broadly elliptic stigma in the middle (Fig. 4A). Because Klebs (1896) depicted an anterior stigma in the type illustration, strain HcCl-5-3 is here assigned to *C. media* while SAG 10.87 should be redetermined. Under light microscopy, SAG 10.87 was similar to *Chlamydomonas terricola* Gerloff, but it is currently treated as a synonym of *Chlamydomonas noctigama* Korshikov based on the molecular phylogeny of strain UTEX 406 (Buchheim *et al.* 1997), which had been identified as *C. terricola* (Starr 1964). Therefore, for conclusive identification of strain SAG 10.87, further comparative studies are necessary.

Conclusion

Two species of *Chlamydomonas*, *C. pseudomacrostigma* and *C. media*, were re-characterized using detailed microscopy, and their phylogenetic positions were revealed for the first time using 18S *r*RNA genes. Both species were clearly shown to belong to the clade *Monadinia*. Because the type species of *Chlamydomonas*, *Chlamydomonas reinhardtii* P. A. Dang., belongs to the clade *Reinhardtinia* (Nakada *et al.* 2008, Nakada *et al.* 2010b), the species in clade *Monadinia* should be excluded from *Chlamydomonas* after detailed characterizations of the other species/strains.

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References

Andersen, R. A., J. A. Berges, P. J. Harrison & M. M. Watanabe. 2005. Appendix A: Recipes for freshwater and seawater media. *In*: Andersen, R. A. (ed.), Algal Culturing Techniques, pp. 429–538. Elsevier, Amsterdam.

Boldina, O. N. 1996. The main types of ultrastructure of the pyrenoids in *Chlamydomonas* (*Chlorophyta, Chlamydomonasdales*). Bot. Z. 81: 49–58. (in Russian)

Boldina, O. N. 2011. Classification principles of pyrenoids in green monads. *In*: Patova, E. V. (ed.), The Algae: Taxonomy, Ecology and Use in the Monitoring, pp. 34–40. Ural Div. of Russian Academy of Sciences, Yekaterinburg. (in Russian)

Bourrelly, P. 1951. Volvocales rares ou nouvelles. Hydrobiologia 3: 251–281.

Buchheim, M. A., J. A. Buchheim & R. L. Chapman. 1997. Phylogeny of the VLE-14 *Chlamydomonas* (Chlorophyceae) group: a study of 18S rRNA gene sequences. J. Phycol. 33: 1024–1030.

Buchheim, M. A., C. Lemieux, C. Otis, R. R. Gutell, R. L. Chapman & M. Turmel. 1996. Phylogeny of the Chlamydomonadales (Chlorophyceae) a comparison of ribosomal RNA gene sequences from the nucleus and the chloroplast. Mol. Phylogenet. Evol. 5: 391–402.

Buchheim, M. A., M. Turmel, E. A. Zimmer & R. L. Chapman. 1990. Phylogeny of *Chlamydomonas* (Chlorophyta) based on cladistic analysis of nuclear 18S rRNA sequence data. J. Phycol. 26: 689–699.

Cantino, P. D. & K. de Queiroz. 2010. PhyloCode: International Code of Phylogenetic Nomenclature, Version 4c.

Ettl, H. 1976. Die Gattung *Chlamydomonas* Ehrenberg. Beih. Nova Hedwigia 49: 1–1122.

Ettl, H. 1983. Süßwasserflora von Mitteleuropa, vol. 9, Chlorophyta I. Phytomonadina. Gustav Fischer, Stuttgart.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using bootstrap. Evolution 39: 783–791.

Gerloff, J. 1940. Beiträge zur Kenntnis der Variabilität und Systematik der Gattung *Chlamydomonas*. Arch. Protistenkunde. 94: 311–502.

Kato, S. 1982. Laboratory culture and morphology of *Colacium vesiculosum* Ehrb. (Euglenophyceae). Jap. J. Phycol. 30: 63–67. (in Japanese)

Klebs, G. 1896. Ueber die Fortpflanzungs-Physiologie der niederen Organismen, der Protobionten, Specieller Theil, Die Bedingungen der Fortpflanzung bei einigen Algen und Pilzen. Gustav Fischer, Jana.

Konstantinova, I. A. & O. N. Boldina. 2000. Comparative analysis of the pyrenoid ultrastructure in green monad and coccoid algae. Russ. J. Pl. Physiol. 47: 655–659.

Korshikov, O. A. 1938. Vyznachnyk prisnovodnykh vodorosteĭ URSR, IV, Volvocineae. Vydavnyt͡stvo Akademiï Nauk, URSR, Kiev. (in Ukrainian)

Massjuk, N. P., G. G. Lilitska & D. O. Kapustin. 2011. Chlamydomonadales. *In*: Tsarenko, P. M., S. P. Wasser & E. Nevo (eds.), Algae of Ukraine: Diversity, Nomenclature, Taxonomy, Ecology and Geography, vol. 3, Chlorophyta, pp. 157–218. A. R. A. Gantner, Ruggell.

Nakada, T., K. Misawa & H. Nozaki. 2008. Molecular systematics of Volvocales (Chlorophyceae, Chlorophyta) based on exhaustive 18S *r*RNA phylogenetic analyses. Mol. Phylogenet. Evol. 48: 281–291.

Nakada, T. & H. Nozaki. 2007. Two species of *Chlamydomonas* (Volvocales, Chlorophyceae) new to Japan. J. Jap. Bot. 82: 179–189.

Nakada T. & H. Nozaki. 2009. Taxonomic study of two new genera of fusiform green flagellates, *Tabris* gen. nov. and *Hamakko* gen. nov. (Volvocales, Chlorophyceae). J. Phycol. 45: 482–492.

Nakada, T., H. Shinkawa, T. Ito & M. Tomita. 2010b. Recharacterization of *Chlamydomonas reinhardtii* and its relatives with new isolates from Japan. J. Pl. Res. 123: 67–78.

Nakada, T., T. Soga & M. Tomita. 2010a. Phylogenetic position of a rare loricated green alga, *Cephalomonas granulata* N. L. Higinb. (Volvocales, Chlorophyceae). Phycol. Res. 58: 62–68.

Nakada, T., S. Suda & H. Nozaki. 2007. A taxonomic study of *Hafniomonas* (Chlorophyceae) based on a comparative examination of cultured material. J. Phycol. 43: 397–411.

Nakazawa, A. & H. Nozaki. 2004. Phylogenetic analysis of the tetrasporalean genus *Asterococcus* (Chlorophyceae) based on 18S ribosomal RNA gene sequences. J. Jap. Bot. 79: 255–261.

Nozaki, H., K. Misawa, T. Kajita, M. Kato, S. Nohara & M. M. Watanabe. 2000. Origin and evolution of the colonial Volvocales (Chlorophyceae) as inferred from multiple, chloroplast gene sequences. Mol. Phylogenet. Evol. 17: 256–268.

Nylander, J. A. A. 2004. MrModeltest 2.2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Sweden.

Pascher, A. 1927. Die Süßwasser-Flora Deutschlands, Österreichs und der Schweiz, Heft 4, Volvocales = Phytomonadinae, Flagellatae IV = Chlorophyceae I. Gustav Fischer, Jana.

Péterfi, L. S. 1968. Studies on the taxonomy and ecology of the Romanian Volvocales. II. Nova Hedwigia 16: 215–249.

Posada, D. & K. A. Crandall. 1998. Modeltest: testing the model DNA substitution. Bioinformatics 14: 817–818.

Pringsheim, E. G. 1946. Pure Cultures of Algae. Cambridge University Press, Cambridge.

Pröschold, T. & F. Leliaert. 2007. Systematics of the green algae: conflict of classic and modern approaches. *In*: Brodie, J. & J. Lewis (eds.), Unravelling the algae: The Past, Present, and Future of Algae Systematics, pp. 123–153. CRC Press, Boca Raton.

Pröschold, T., B. Marin, U. G. Schlösser & M. Melkonian. 2001. Molecular phylogeny and taxonomic revision of *Chlamydomonas* (Chlorophyta). I. Emendation of *Chlamydomonas* Ehrenberg and *Chloromonas* Gobi, and description of *Oogamochlamys* gen. nov. and *Lobochlamys* gen. nov. Protist 152: 265–300.

Ronquist, F. & J. P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.

Rosowski, J. R. & R. W. Hoshaw. 1988. Advanced anisogamy in *Chlamydomonas monadina* (Chlorophyceae) with special reference to vacuolar activity during sexuality. Phycologia 27: 494–504.

Saitou, N. & M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406–425.

Starr, R. C. 1964. The culture collection of algae at Indiana University. Amer. J. Bot. 51: 1013–1044.

Swofford, D. L. 2002. PAUP*: Phylogenetic Analysis Using Parsimony (* and Other Methods). Version 4.0b10. Sinauer, Sunderland, MA, USA.

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